AD 407 017

ALASKAN AIR COMMAND
ARCTIC AEROMEDICAL LABORATORY
FORT WAINWRIGHT

(4) METABOLISM OF FATTY ACIDS AND RELATED SUBSTANCES IN ANIMALS EXPOSED TO COLD,

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TECHNICAL NOTE
(18) AAL-TN-61-5
(19)

(11) OCTOBER 1961

METABOLISM OF FATTY ACIDS AND RELATED SUBSTANCES IN ANIMALS EXPOSED TO COLD *

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Further Studies on the Inhibitor of Lipogenesis Induced by Fasting at Normal Room Temperature and at Low Environmental Temperature

In ~~the last report (Rapport,~~ 1961) evidence was presented for the fact that the microsomes of the liver from animals fasting at 25° C or 0° to 2° C are inhibitory to the lipogenic activity of the liver cell. It was further shown that sonic treatment of these microsomes liberated, in a water soluble form, an inhibitory material which mimicked that of the microsomes from these cells. Chromatography on Sephadex G25 indicated that this inhibitory material was of a colloidal nature. For this reason it was decided to investigate further the colloidal nature of this substance by the process of dialysis, ~~(Table I).~~ It can be seen that this microsomal inhibitor of lipogenesis is non-dialyzable and presumably, therefore, is either a colloid or a substance tightly bound to a colloid.

TABLE I

Enzyme	Inhibitor	mμmoles acetate-1-C^{14} conv. to fatty acid
Fed Supernatant	None	300
Fed Supernatant	Fasted microsome sonicate supernatant	170
Fed Supernatant	Dialysed fasted microsome sonicate supernatant	180

In the last report (Rapport, 1961) we stated that the material was labile and that heating for 10 minutes at 100° C completely destroyed it. We decided to characterize further this heat lability by studying the effect of heating for 15 min. at 60° C, and as can be seen from Table II this treatment almost completely destroyed the lipogenic inhibitor.

* Studies covering period 1 November 1960 ~~through~~ 31 January 1961.

TABLE II

Enzyme	Inhibitor	mμmoles Acetate-1-C^{14} Conv. to Fatty Acid
Fed Supernatant	None	255
Fed Supernatant	Fasted microsome sonicate supernatant	25
Fed Supernatant	Fasted microsome sonicate supernatant heated at 60° C for 15 min.	210

Observing such heat lability in a colloidal substance would seem to suggest that the material is a protein, and for this reason we decided to study the material by means of ammonium sulfate fractionation (Table III). It can be seen that most of the inhibitory material is precipitated by ammonium sulfate in the fraction that precipitates between 30% and 50% saturation.

TABLE III

Enzyme	Inhibitor	mμmoles Acetate-1-C^{14} Conv. to Fatty Acid
Fed Supernatant	None	230
Fed Supernatant	Fasted microsome supernatant	45
Fed Supernatant	0% to 30% $(NH_4)_2SO_4$ fract. of fasted microsome sonicate supernatant	145
Fed Supernatant	30% to 50% $(NH_4)_2SO_4$ fract. of fasted microsome sonicate supernatant	70
Fed Supernatant	50% to 100% $(NH_4)_2SO_4$ fract. of fasted microsome sonicate supernatant	215

This finding is quite reproducible if care is taken to make sure that the pH of the system is 7 and that the salt concentration aside from ammonium sulfate is negligible and that the temperature is constant at around 0° C. Any deviation from these conditions leads to rather great shifts in the precipitability of the material. On the basis of the dialysis and sephadex G25 studies as well as the heat lability and the ammonium sulfate fractionation studies, it was concluded that the inhibitor is almost surely a protein. For this reason further purification of this protein was attempted by use of the DEAE column chromatography procedure. Because of the fact that this chromatography procedure involves elution with aqueous solutions of high ionic strength, it seemed desirable to study the effect of high ionic strength itself on the lipogenic system of the supernatant of the liver cell. As can be seen from Table IV a high ionic strength greatly affects the ability of the supernatant system of liver to convert acetate to fatty acids, and for this reason it was necessary to dialyze all of the DEAE chromatographic fractions to prevent the microsomal inhibitory action from being masked by the inhibitory effect of the high ionic strength.

TABLE IV

Enzyme	Ionic Strength of Medium	mμmoles Acetate-1-C^{14} Conv. to Fatty Acid
Fed Supernatant	0.05	325
Fed Supernatant	0.37	90

This fact, of course, has made the DEAE column chromatography very laborious. However, work has been carried out with it and has led, on the basis of batch chromatography and column chromatography, to the tentative conclusion that DEAE cellulose does not adsorb the protein inhibitor of the microsomes. This cellulose can be used as the first step in purification by removing many contaminating proteins from the inhibitory protein. Further work is at the present time in progress on other cellulose preparations in order to find a tool for purifying the inhibitor, and starch gel electrophoresis is also being considered. It is hoped by these electrophoretic and chromatographic methods that in the next three months the further purification of this inhibitory protein will have been an accomplished fact.

In regard to the biochemical mechanism of action of this inhibitory material on lipogenesis, it of course would be most desirable to deal with a pure substance. However, certain preliminary studies have been made with the supernatant of the sonicated microsomes and have lead to some interesting results. The possibility

that the substance acts by means of an ATPase activity seems quite possible since the lipogenic system used in our studies is ATP-dependent. It was felt that some insight could be gained on this by increasing the amount of ATP added to the lipogenic system, and in Table V the effects of doubling the ATP concentration from the usual 3 mM to a 6 mM level are recorded. It is obvious from this table that ATP itself in concentrations twice that of the 3 mM (experimentally determined optimal concentration for lipogenesis by the supernatant) is inhibitory. It is further seen that once the ATP concentration is doubled the addition of the microsomal sonicate supernatant is not inhibitory. It might be concluded from these

TABLE V

Enzyme	ATP (Sodium Salt) mM	Inhibitor	mμmoles Acetate-1-C^{14} Conv. to Fatty Acid
Fed Supernatant	3	None	315
Fed Supernatant	6	None	115
Fed Supernatant	3	Fasted microsome sonicate supernatant	105
Fed Supernatant	6	Fasted microsome sonicate supernatant	115

results that our inhibitor is an ATPase, but considerable caution must be used because of the fact that at the higher levels the ATP itself so limits the rate of lipogenesis that the inhibitor is unable to reduce it further. Therefore one cannot consider these results with the ATP addition as anything other than suggestive. It was of considerable interest that ATP did cause this inhibition of lipogenesis when lifted from 3 mM to 6 mM, and we wondered if this effect was due to the fact that the ATP used was the disodium salt. For this reason similar studies were carried out with the dipotassium salt, and as can be seen from Table VI the dipotassium salt is as inhibitory as the disodium salt. From the ionic strength studies carried out in this laboratory, it is not likely that the inhibitory action of ATP is the result of its ionic strength since it adds very little total ionic strength to the whole incubation system. It would, therefore, seem that ATP in its own right is in some way responsible for the effect. This ATP action brings to mind the fact that it has been shown that 3', 5' cyclic AMP is ketogenic. It is usually the case that a ketogenic substance is also a lipogenic inhibitor, and it seemed

TABLE VI

Enzyme	ATP (Potassium Salt) mM	mμmoles Acetate-1-C^{14} Conv. to Fatty Acid
Fed Supernatant	3	175
Fed Supernatant	6	55

entirely conceivable that our inhibitor and the ATP in excess could be working by means of generating 3', 5' cyclic AMP, which in turn would inhibit lipogenesis. For this reason studies were carried on by directly adding 3', 5' cyclic AMP to the incubation system and the results of this study are recorded in Table VII. As can be seen from these data, 3', 5' AMP is without effect. Another way in which

TABLE VII

Enzyme	3', 5' cyclic AMP (mM)	mμmoles Acetate-1-C^{14} Conv. to Fatty Acid
Fed Supernatant	0	280
Fed Supernatant	200	270
Fed Supernatant	20	270

the inhibitory protein could limit lipogenesis would be by the process of binding biotin, which is necessary for the acetyl carboxylase step in the lipogenic system. It was felt that such binding of biotin by the inhibitor could be overcome by having the inhibitor react with exogenous biotin and in this way preventing it from reducing lipogenesis by binding the endogenous biotin present. The first experiment that we did on this was positive, but since that initial experiment we have been unable to confirm this finding in spite of repeated attempts. Not only have we done the experiments in which biotin was added as in the original positive experiment, but we have done pre-incubation system experiments with the inhibitor and the biotin; not one of the subsequent experiments has shown any ability of the biotin to overcome the inhibitory action of the microsomal inhibitor induced by fasting. Since this hypothesis is attractive and since positive evidence for it was found once, we are still trying to design experiments that might establish the effect.

Another possibility by which this inhibitor from the microsomes of the fasted animal could limit lipogenesis is by altering the pH of the system during the incubation period, and for this reason we decided to study the pH changes occurring during the incubation period. It was found that, whether the inhibitor as the water-soluble sonicate supernatant of microsomes of fasting animals or the microsomes from fasting or cold-fasting animals or no inhibitor is added, the pH of our incubation system did not change, remaining near to 7.0 throughout the incubation period.

On the basis of data presented in the last progress report (Rapport, 1961), it is clear that, although the fasting microsomes have a great inhibitory action on lipogenesis, a moderate inhibition was also found with microsomes from the fed animals. It was felt that this might be due to the fat content of the diet of these animals since it is well known that fat in the diet limits lipogenesis. For this reason we decided to study a rat fed a fat-free diet very high in carbohydrate for one week (a diet known to promote lipogenesis greatly). However, to our surprise, the microsomes of the animals fed the fat-free high carbohydrate were more inhibtory than the microsomes from the animal fed the fat-containing diet (Table VIII), although microsomes from both fed groups were not nearly as inhibitory as microsomes from fasted rats. It should also be noted, however, that the supernatant of

TABLE VIII

Enzyme	Particles	mμmoles Acetate-1-C^{14} Conv. to Fatty Acid
Supernatant	None	570
Supernatant	Microsomes	225

the rats fed the fat-free diet has a far higher lipogenic activity than supernatant from rats fed a fat-containing diet. Therefore, it would seem that the lipogenic inhibitory action of dietary fat is probably not acting by generating a microsomal inhibitor but in some other way. It is known that adding glucose (about 5 g to a 250 g animal) by stomach tube and then sacrificing the animal a few hours later leads to a very high lipogenic activity in liver slices prepared from that rat. This is sometimes called superlipogenesis. We decided, therefore, to see if the effect of adding glucose by stomach tube to the animal and thus producing superlipogenesis in its liver would completely remove the small amount of lipogenic inhibition seen in the microsomes from the animal fed the standard fat-containing diet. It can be seen from Table IX that the stomach tubing of an animal with glucose prior to sacrifice still resulted in the microsomes having a small but definite inhibitory activity on supernatant lipogenesis.

TABLE IX

Enzyme	Particles	mμmoles Acetate-1-C^{14} Conv. to Fatty Acid
Supernatant	None	330
Supernatant	Microsomes	295

So far we have been unable, therefore, to find any condition where the microsomes of the liver have no inhibitory activity whatsoever. We intend to continue our studies with the physiological conditions which induce or prevent the presence of an inhibitor in the microsomes and are interested at the present time in studying the effect of insulinizing a normal animal.

Effects of Cold Acclimation on Hepatic Lipogenesis

This laboratory has reported that cold acclimation causes a marked reduction in hepatic lipogenesis, the work being done with liver slices prepared from cold-acclimated rats. Subsequently experiments were carried out with liver homogenates, and in these homogenates in which ATP generation and TPN generation were made optimal, no defect in lipogenesis from acetate could be noted. These discrepancies between the liver slices and the homogenate have been difficult to unravel completely, but it looked as if the liver homogenate from cold-acclimated animals was being supplied the exogenous cofactor environment necessary for lipogenesis which was not available to the intact liver slice itself. Therefore the general conclusion was drawn that the lipogenic inhibition in the liver of the cold-acclimated animal probably is related to a lack of the proper concentration of some cofactor involved in lipogenesis. The exact nature of this cofactor has been explored, but so far no definite answer has been obtained. The work on liver homogenate systems is done with a one-hour incubation period since it was shown earlier that the rate of lipogenesis in control rats is linear during this time. It was noted with the liver homogenates from the cold-acclimated rats that, although statistically no difference in lipogenic activity could be established between homogenates prepared from cold-acclimated and those prepared from control rats, the homogenates from cold-acclimated rats did seem to incorporate somewhat less acetate into fatty acids than did the ones from control rats. We therefore decided to see if the above-noted linearity in control rats also applied to the cold-acclimated ones. The results showed that the lipogenic activity of the homogenates from the cold-acclimated rats fell during the last half hour of the incubation period, indicating a greater lability of the enzyme system. This will be explored further. It is of course very difficult to relate lability of homogenates to an intracellular defect, but we feel that work on the matter may lead to a better understanding of the problems in lipogenesis induced by cold acclimation.

Effect of Cold Acclimation on Palmitate Metabolism by the Intact Rat

In the last report we presented a considerable amount of data on the metabolism of palmitate in normal and cold-acclimated rats fed fat-free diets. In the present report we should like to present similar experiments on the metabolism of palmitate by animals fed a standard diet containing about 50% carbohydrate, 25% protein and 10% fat. The labeled palmitate was administered in the same way, as in the last report (i. e., in the presence of an abundance of glucose). In Table X are recorded the per cent of this administered palmitate that was deposited in the whole rat as esterified fat. A breakdown of fat deposition into some representative tissues of the rat is also reported. It can be seen that in the case of both the 25° C acclimated rat and the 0° to 2° C acclimated rat approximately the same amount of fed palmitate is laid down in the body fat. Apart from the brown fat, in which the cold-acclimated deposited more of the labeled fat, the other tissues studied

TABLE X

Physiol. State and Tissue	% Palmitate-1-C^{14} Present
25° C Acclimated Rat	
Whole rat	41.6
Liver	3.3
Epididymal fat pad	0.9
Lumbar adipose tissue	1.5
Interscapular brown fat	0.6
0° to 2° C Acclimated Rat	
Whole rat	39.1
Liver	2.3
Epididymal fat pad	0.4
Lumbar adipose tissue	0.6
Interscapular brown fat	1.4

deposited less. The white adipose tissue analyzed accounted for about 5% of the total fat laid down. Unless the fat sampling was not representative, it appears some other tissues of the cold-acclimated animal must accumulate considerably more of the esterified palmitate than the controls. The brown fat, of course, is one of these tissues, but certainly not the only one. Further insight on this problem is gained from specific activity measurements on the various tissues and the whole rat of both cold-acclimated and 25° C acclimated animals. These data are

TABLE XI

Physiol. State and Tissue	Specific Activity of Fatty Acids (cts/min/mg)
25° C Acclimated Rat	
Whole rat	322
Liver	2380
Epididymal fat pad	100
Mesenteric fat	167
Perirenal fat	170
Interscapular brown fat	1640
0° to 2° C Acclimated Rat	
Whole rat	495
Liver	1373
Epididymal fat pad	99
Mesenteric fat	201
Perirenal fat	203
Interscapular brown fat	1693

recorded in Table XI. The higher specific activity in the whole rat in the case of cold-acclimated animals is merely the reflection of a somewhat lower fat content than in the 25° C acclimated animal, since both animal groups laid down approximately the same amount of fat in their body. The specific activity of both the white and brown adipose tissue is about the same in the two groups, while the specific activity of the control livers was almost twice as great as that of the cold-acclimated livers. It can be seen in Table X that the cold-acclimated rat incorporated far more of the palmitate into this brown adipose tissue pad than did the animal that was acclimated to 25° C. Therefore, this high incorporation is merely a reflection of the fact that the cold-acclimated animal has a very large brown adipose tissue mass, there being no difference in the affinity of the brown fat for palmitate in the two groups. It should be noted that in both animal groups the interscapular fat pad has a very high affinity for the accumulation of the palmitate, which is shown by the high specific activity in comparison with other adipose tissue areas and the liver. When you consider that the fat content of the liver is far less than that of the interscapular brown fat pad, it also becomes quite clear that on the basis of protoplasmic mass the interscapular brown fat has a far greater affinity for the palmitate of the diet than does the liver itself.

It is of interest that the specific activity of the various white adipose tissue sources varies markedly (e. g., the lumbar adipose tissue has a much higher

specific activity than the epididymal). These data suggest that there is marked variation in the affinity of adipose tissue for dietary fat. Such metabolic heterogeneity casts some doubt on the value of making generalizations on adipose tissue metabolism on the basis of *in vitro* studies using epididymal fat pads, although currently such generalizations are at the crest of popularity.

Effect of Sonication on Palmitate Activation Activity

In the last report we stated that the activation of fatty acids of the C-12 length and below was not influenced by sonic destruction of mitochondria, but activation of the C-16 acid did increase. This work has been repeated during the past three months with essentially the same findings. It is therefore clear that part of the activating enzyme for the long-chain fatty acids is in some sort of anatomical structural barrier that prevents it from having free access to the long-chain fatty acid substrate even when this substrate is presented as albumin-palmitate. This fact of course causes a serious problem in making a precise measure of palmitate-activating enzyme activity. For this reason we studied the effect of various sonication times on the ability of mitochondria to activate palmitate. These curves indicate that the attainment of reproducible data is going to be difficult to achieve, because the results indicate strongly that there is not only a release of the activating-enzyme activity by sonication but that sonication to some extent also destroys the enzyme itself. We have found that in the case of mitochondria the optimal activating activity is somewhere around 10 to 20 minutes of sonication, but there is no proof that this optimal time is anything other than the algebraic sum of two opposing forces, the exposure of the enzyme by destroying mitochondrial structure vs. the destruction of the enzyme itself by the sonication. For this reason it is extremely difficult with our techniques ever to measure the true palmitate-activation enzyme activity of mitochondria. Similar studies were carried out with total homogenates, and again sonication was found to cause an increase in the activity of the palmitate-activation enzyme. In these homogenates an optimal sonication time was found to be about 10 minutes. Here again, however, this is merely the time in which the balance of forces between destruction of the mitochondria and the destruction of the enzyme favor the former. It is hoped that in subsequent work we will find methods of exposing the mitochondrial enzyme that will not be destructive to the enzyme in its own right. Similar experiments to those were carried out with microsomes, and it was found that they (previously shown to contain very high palmitate activation activity) are not affected by the sonication technique. It therefore seems that the microsomal palmitate-activating enzyme is not compartmentalized in such a way that it is incompletely available to the palmitate-albumin substrate.

The Effect of Cold Exposure and Fasting on Palmitate-Activating Enzyme Activity

In the last report we stated that liver homogenates prepared from cold-fasted rats have the same ability to activate palmitate as did the liver homogenates

prepared from control rats. However, in these studies the homogenate was used as such (i. e. was not sonicated), and therefore the enzyme activities measured in both groups were affected by compartmentalization; thus a true measure of the palmitate-activating activity was not measured. As stated above, it is impossible actually to get a true value of this activity, but with the sonication technique a reasonably good approximation of the true value can be made. We therefore repeated the work reported last time on sonicated homogenates prepared from cold-fasted and control rats. No difference was found in the ability of these sonicated homogenates to activate palmitate, and for this reason we feel that the palmitate-activating activity (the true activity) is approximately the same in the case of the liver of both the cold-fasted and control rats.

Intracellular Distribution of Activating Enzyme Activity of Various Chain Length Fatty Acids

In our last report we stated that the activation of fatty acids of chain lengths from C-4 to C-18 had quite different intracellular distribution than the activation of acetate. Specifically, acetate was activated primarily by the supernatant fraction of the cell and to some extent by the mitochondria, but no activating activity was found in the microsomes. In contrast to this, the C-4 to C-18 acid seemed to be activated primarily in the microsomes and to a reasonable extent by the mitochondria. However there was little or no activating activity in the supernatant. This difference surprised us, and we felt that it should be examined more carefully by using higher homogenate concentrations so that we might detect the amount of activating enzyme in the supernatant for C-4 to C-18 acids which was masked by diluting the homogenate to the low level of 10% as was done in the previous reports. However, when we used supernatant for 20% homogenates the blanks that we obtained with the supernatant were in many cases considerably higher than that of the system to which the albumin-palmitate substrate was added. It was felt that this probably was due to the fact that endogenous substrates were being activated and that the fatty acids were in some way blocking the activation of the endogenous substrate, thus making the test value lower than the blank value in the assay. The difficulty, we felt, could possibly be eliminated by removing much of the endogenous substrate by means of Sephadex G-25 column chromatography. This procedure was carried out, and the crystalloid contaminants were eliminated, which to a great extent reduced--but did not completely remedy--the problem of high blanks. On assaying for the C-4 through C-12 acids we observed no activity at all when using supernatants from reasonably high concentrations of homogenate. We must therefore conclude, barring some unknown interference of the assay in the supernatant of the cell, that unlike the case of acetate, activation of longer-chain fatty acids, starting with C-4, does not occur in the supernatant of the cell. This difference in activating activity is indeed a puzzling one and certainly merits considerable thought and probably further investigation.

REFERENCES

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